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SECOND TRIANNUAL REPORT (YEAR 1)

for period January 1, 1994 to May 31, 1994

Report Date: June 17, 1994

ONR Grant No. N00014-93-J-1034
(ECU Grant #5-01071)

PRECLINICAL INVESTIGATION OF LYOPHILIZED PLATELET PREPARATIONS

Principal Investigator:

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School of Medicine

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Attachments:

1. Report from subcontract principal investigator, Marjorie S. Read, Ph.D., The University of North Carolina at Chapel Hill.
2. Abstract submitted to American Association of Blood Banks
3. Infusion Study Summaries 1 - 3

94-19361



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Administrative Activity:

We have experienced delays in two project areas, one of which has been overcome. The Xylum Clot Signature Analyzer (CSA) for in vitro testing of hemostatic capacity of the lyophilized platelets was listed as an item for purchase at the inception of the grant under Specific Aim #4. However, production of the commercial model was delayed by the manufacturer, who agreed instead to rebuild a prototype for us to use until a production model became available. The PI was trained on the prototype in March during its final operational checkout, and it was delivered in April. We have now begun use of the CSA in the studies of lyophilized platelet infusions in dogs on clinical heart-lung bypass as a test of hemostasis. The other delay occurred in carrying out the gel studies of activation signals in stimulated lyophilized platelets. The technician (Jenny Potter) in the co-investigator's laboratory (Charles Knupp, M.D.) had to take an extended leave of absence for a serious health problem just after completing the set-up work. There is no other technician in that laboratory to fill in. At present, we are trying to identify other local laboratories with the necessary expertise at ECU or UNC-Chapel Hill. However, on a positive note, alliance of our efforts with Armour Pharmaceutical Corporation will greatly advance progress on viricidal/bacteriocidal effects of the stabilization process as detailed under Specific Aim #3.

An abstract was generated and submitted to the American Association of Blood Banks (see Attachment 2) with data from the first lyophilized platelet infusion studies carried out at ECU (along with data from the Xylum CSA for other related purposes: see grant report for same period on ONR Grant #N00014-92-J-1244). On May 18, there was an ATD review conference held at the Naval Research Laboratory in Alexandria, VA, to discuss progress on this project and related ones from other participants. Our data presented at this conference included the content of the AABB abstract and appeared to be well received by the peer group present.

The initial budgeting of ECU personnel salaries and benefits was not correctly assigned on the account books at the outset of this grant. This situation has been clarified with the departmental administrators involved and appears to be corrected. The apparent lag of expenditures in this project budget reflects this oversight rather than a lack of research activity.

Scientific Progress:

Start-up of the canine infusion studies at ECU was delayed for six months in waiting for delivery of the Xylum CSA since it provides a precise method of evaluation of the effect of infused platelets. Even so, three such studies were carried out in the absence of the CSA to generate preliminary results on the integrity of the model system. Instead, data was collected on the ear bleeding time as planned along with a jury-rigged vessel puncture bleeding time in the jugular vein. In preparation for this study, one full-time technician was dedicated to the task of establishing the processing of canine lyophilized platelets to provide enough material to infuse the equivalent of 2/3 of the platelet mass of the recipient subject animals. Twenty-two different preparations have been made so far with adjustments along the way until yields were

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improved sufficiently to make the necessary amount of infusate from a minimal number of donor animals. For the first infusion study, 247×10^9 platelets were infused, and 288×10^9 platelets in the second study. Attached to this report are the bleeding time and platelet count results showing the effectiveness of infused lyophilized platelets in lowering the bleeding times prolonged by heart-lung bypass versus a control dog receiving no such infusion. We observed that the reduction in bleeding times was not apparent immediately, but at least by 20 minutes post-infusion, and became more remarkable after the animal was removed from heart-lung bypass. The platelet count increments showed also that 50-90% of the lyophilized platelets were in circulation an hour after infusion.

More experiments are underway to extend this test of the hemostatic capabilities of lyophilized platelets. Other controls include infusion of canine plasma alone or in combination with lyophilized platelets to correct the bleeding, fresh canine platelets, and fresh canine platelets stabilized and washed but not freeze-dried. We plan also to infuse the lyophilized platelets at smaller doses to determine the minimal effective dose, and at other stages in the procedure (during weaning from the pump). All of these studies will benefit from having the Xylum CSA in place to provide more useful information than in vivo bleeding times alone.

Report period: January 1994- May 1994
University of North Carolina at Chapel Hill

Contract: UNC/ECU
Grant No. N00014-93-1034
The Office of Naval Research
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Performance Site: University of North Carolina at Chapel Hill
Principal Investigator: Marjorie S. Read, Ph.D

Grant No. N00014-93-1034

Studies conducted January 1994- May 1994

The biochemical and thrombogenic effects of rehydrated platelets and the characterization of the surface antigens of rehydrated platelets (Specific aim #1)

We are continuing our studies of rehydrated human and canine platelets using SDS-PAGE and immunoblotting of human and canine platelet lysates and releasates. Lysates and releasates of rehydrated platelets were prepared as previously described (see progress report for October 1993-December 1993). Commercially available antibodies were used to probe Western blots of lysates and releasates for von Willebrand factor (vWF) (DAKO), actin (Sigma Immunochemicals), thrombospondin (AMAC, Inc.), GPIb (DAKO), fibrinogen (Calbiochem Corp.), and fibronectin (Calbiochem Corp.).

The vWF antibody reacted with a high molecular weight protein band present in western blots of both fresh and rehydrated human and canine platelets. The actin antibody reacted with a low molecular weight band present in western blots of both fresh and rehydrated platelet lysates and releasates (both human and canine). The thrombospondin antibody did not react well with any protein in the rehydrated platelets, and only minimally reacted with a protein in the fresh platelet preparation. The antibody to GPIb reacted minimally with a high molecular weight protein on Western blots of fresh platelets without any visible reaction with Western blots of rehydrated platelet preparations. The antibody to fibrinogen reacted strongly with several protein bands (perhaps non specifically) in Western blots of fresh platelets, with minimal reactivity in Western blots of rehydrated platelets. The antibody to fibronectin reacted with a high molecular weight protein in western blots of fresh platelets, but did not react with any proteins in Western blots of rehydrated platelets. These experiments are being repeated with the Amersham ECL chemiluminescence system to enhance the sensitivity of the assays. We suspect that a more sensitive detection system will reveal reactivity of some of these antibodies with rehydrated platelet preparations on Western blot.

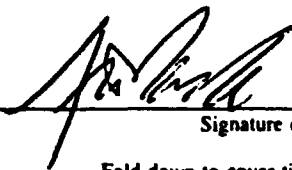
We have also begun the characterization of human and canine rehydrated platelets using light microscopy. For these studies, rehydrated platelets and fresh platelets are allowed to spread on a glass slide for 5 minutes-2 hours. The spread platelets are then fixed onto the slide and treated with detergent to permeabilize the cell membrane. For the visualization of filamentous actin within the platelets, FITC- or TRITC-labeled phalloidin is incubated with the platelets. For the visualization of other platelet proteins, antibodies specific for these proteins are incubated with the platelets. A secondary antibody labeled with fluorescein or rhodamine is used to visualize the localization of the antibodies in the platelets. We have used TRITC-phalloidin (Molecular Probes) and FITC-phalloidin (Sigma Immunochemicals) for labeling filamentous actin. Antibodies to GPIb (DAKO), GPIIb/IIIa (AMAC, Inc.), myosin (Sigma Immunochemicals), and tropomyosin (Sigma Immunochemicals) are also being used. Preliminary data suggests that

actin arrangement in rehydrated platelets is similar to that seen in fresh platelets. In fresh and rehydrated platelets fixed onto the slide after 5 minutes, actin staining appears at the center of the discoidal platelets. In normal platelet spreading, filopodia extend from the platelet with actin filaments staining the length of the filopodia. In a comparison of fresh platelets and rehydrated platelets, filopodia formed at a faster rate in fresh platelets than in rehydrated platelets. However, actin staining within the filopodia of fresh and rehydrated platelets was similar. Antibodies to GPIb and GPIIb/IIIa reacted with a protein on the surface of platelets from fresh and rehydrated platelet preparations. The staining pattern for these proteins appears to be similar in fresh and rehydrated platelets throughout the spreading process. Antibodies to myosin and tropomyosin are also being used for these experiments, and preliminary data will be available soon. Although these experiments are currently being used to characterize the rehydrated platelets, they might lead to the future development of a quality control test for rehydrated platelet preparations.

The effects of multiple infusions of rehydrated platelets in the canine animal model (Specific aim #2)

Multiple transfusions of rehydrated platelets has not led to a significant increase in the immune response of transfused animals. We have used an ELISA to determine if any animals that have received rehydrated platelets has developed an antibody to the rehydrated platelets. Our data suggests that one of the three dogs that have received multiple infusions of rehydrated canine platelets has developed an antibody to platelets from 2 of the 3 canine (platelet) components of the infusate pool. We are in the process of determining if the antibodies were generated to a moiety specific to rehydrated platelets, or if they were generated to a moiety contained in both fresh and rehydrated platelets.

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HEMOSTATIC PROPERTIES OF LYOPHILIZED PLATELETS IN TESTS OF BLEEDING TIMES. Arthur P. Bode, Marjorie S. Read, Robert M. Lust, Depts. of Pathology and Surgery, East Carolina Univ., Greenville, NC, and Dept. Pathology, Univ. of N.C. at Chapel Hill.

Background: We have previously shown that lyophilized platelets (L-Plt) retain properties of adhesion and activatability in the Baumgartner perfusion chamber (Trans 33:72S, 1993). Now we have analyzed L-Plt in two systems directly testing hemostatic function. **Study Design:** One is a prototype device simulating the Ivy bleeding time in vitro (IVBT) and collagen-induced thrombus formation (CITF) in recalcified whole blood (Xylum Clot Signature Analyzer: CSA); the other is an in vivo bleeding time in dogs on full clinical heart-lung bypass before and after infusion of L-Plt. **Results:** On the CSA, L-Plt gave an average ($n=4$) IVBT of 1 min 58 sec and a CITF of 73% versus 2 min 14 sec and 88% respectively for fresh platelets. Expired platelet concentrates gave indeterminate results because aggregates clogged the lines. IVBT > 6 min and CITF < 25% is typical of vWD patients. In two canine heart-lung bypass studies, the in vivo bleeding time improved from >15 min to 5-7 min after infusion of a bolus of $2-3 \times 10^{11}$ L-Plt. The corrected count increments were (#1) 88% and (#2) 47% based on estimated circulatory volume. **Conclusions:** These results demonstrate the hemostatic activity of L-Plt and their potential value in transfusion medicine.

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PLATELETS IN TESTS OF BLEEDING TIMES.

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SUMMARY OF NAVY INFUSION STUDY #1 (940318)

EVENT	NEEDLE GAUGE	EAR BLEEDING TIME		NEEDLE GAUGE	VESSEL BLEEDING TIME	
		<u>SLOWED</u>	<u>STOPPED</u>		<u>SLOWED</u>	<u>STOPPED</u>
1. Before heparin, during sternotomy Platelet count = 292,000	25g 23g	- NO BLEEDING - 1 min, 30 sec.	2 min, 15 sec.	25g 23g	----- -----	30 sec 31 sec
2. After heparin, pre-pump Platelet count = 236,000	25g 23g	- NOT DONE - 2 min, 30 sec.	4 min, 48 sec.	25g 23g	----- 1 min	21 sec > 5 min
3. 5 min on pump Platelet count = 25,000	25g 23g	- NO BLEEDING - 5 min, 15 sec	12 - 14 min	25g 23g	- NO BLEEDING - 5 - 6 min	> 8 min
4. 60 min on pump Platelet count = 46,000	25g 23g	5 min, 12 sec 7 min, 20 sec	7 min, 27 sec > 14 min	25g 23g	- NO OUTCOME - 3 min, 30 sec	4 min, 12 sec
5. 120 min on pump Platelet count = 40,000	25g 23g	- NO BLEEDING - 5 min, 20 sec	> 15 min	25g 23g	- NOT DONE - 7 min, 30 sec	> 10 min
LYOPHILIZED PLATELETS INFUSED						
6. 150 min on pump immediately after infusion Platelet count = 124,000 * redo 20 min later, still on pump.	25g 23g	6 min, 30 sec 7 min, 30 sec	8 min, 45 sec > 15 min	25g 23g	- NOT DONE - 8 min	> 10 min
OFF PUMP						
7. Off pump 70 min after infusion Platelet count = 101,000 * redo	25g 23g	3 min, 30 sec 3 min, 40 sec	4 min, 48 sec 5 - 7 min	25g 23g	- NOT DONE - 2 min	2 min, 50 sec
				23g	1 min, 5 sec	1 min, 25 sec

SUMMARY OF NAVY INFUSION STUDY #2 (940504)

EVENT	NEEDLE GAUGE	EAR BLEEDING TIME		NEEDLE GAUGE	VESSEL BLEEDING TIME	
		SLOWED	STOPPED		SLOWED	STOPPED
1. Before heparin, during sternotomy Platelet count = 203,000	25g 23g	34 sec. 1 min, 20 sec.	69, 54 sec. 1 min, 34 sec.	25g 23g	- NOT DONE - -----	31 sec
2. After heparin, before-pump Platelet count = 240,000	23g 23g	1 min 1 min, 30 sec.	1 min, 37 sec. 2 min, 2 sec.	23g	1 min	1 min, 15 sec.
3. 15 min on pump Platelet count = 5,000 Hematocrit = 10	23g 23g (+20 min)	~ 3 min ~ 1 min	> 5 min, indetr ~ 9 min	23g	?	> 5 min, indetr. (rebleeds)
4. 75 min on pump Platelet count = 49,000 Hematocrit = 15	23g	~ 1 min	8 min (oozing)	23g Total blood loss ~ 60 mL	3 min (squirting)	6 min, 40 sec. (oozing)
LYOPHILIZED PLATELETS INFUSED						
5. 120 min on pump 7 min post-infusion Platelet count = 94,000	23g	1 min, 7 sec.	5 min, 37 sec.	23g	1 min	5 min (oozing)
OFF PUMP						
6. 30 min post-infusion Platelet count = 91,000 Hematocrit = 21	23g	1 min, 36 sec.	5 min, 49 sec	23g Total blood loss = 17 mL	~ 1 min	2 min, 10 sec. (oozing)
7. 70 min post-infusion Platelet count = 160,000 Hematocrit = 20	23g	1 min, 40 sec.	indeterminate	23g Total blood loss = 12 mL	1 min	4 min, 30 sec.

SUMMARY OF NAVY INFUSION STUDY #3 (940531)
CONTROL - NO INFUSION

<u>EVENT</u>	<u>NEEDLE GAUGE</u>		<u>EAR BLEEDING TIME</u>		<u>VESSEL BLEEDING TIME</u>	
	<u>SLOWED</u>	<u>STOPPED</u>	<u>SLOWED</u>	<u>STOPPED</u>	<u>SLOWED</u>	<u>STOPPED</u>
1. Before heparin, during sternotomy Platelet count = 479,000	23g	35 sec.	1 min, 04 sec.	23g	30 sec. Blood loss = 2 mL	1 min, 07 sec.
2. After heparin, pre-pump Platelet count = 173,000	23g	46 - 60 sec.	1 min, 14-59 sec.	23g	15 sec. Blood loss = 2 mL	35 sec.
3. 10 min on pump Platelet count = 5,000 Hematocrit = 18	23g	1 min, 46 sec.	2 min, 26 sec.	23g	2 min, 30 sec. Blood loss = 11 mL, other sites oozing.	6 min, 30 sec.
4. 1 hour on pump Platelet count = 27,000	23g	2 min, 20 sec.	6 min, 06 sec.	23g	6 min, 30 sec. Blood loss = 34 mL, spurring until slowed	8 min.
5. 2 hours on pump Platelet count = 23,000	23g	3 min, 40 sec.	10 1/2 - 12 min.	23g	9 min. Blood loss = 80 mL, with old sites oozing also	> 12 min.
<hr/> OFF PUMP - NO INFUSION <hr/>						
6. 10 min. weaning, BP low Platelet count = 24,000	23g	3 min, 50 sec.	9 min.		All old sites re-bleeding (oozing)	
7. 1 hour off pump, BP very low Platelet count = 37,000	23g	3 min, 50 sec.	> 10 min.	23g	After old sites sutured 5 min. Blood loss = 9 mL	6 min, 45 sec.